

**THE EFFECTS OF SIMULATED HYPOGRAVITY ON MURINE
BONE MARROW CELLS**

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ABSTRACT

We have compared mouse bone marrow cells grown in complete medium at unit gravity and a similar population cultured in conditions that mimic some aspects of microgravity. After the cells adjusted to the conditions that simulated microgravity they proliferated as fetal or oncogenic populations; their numbers doubled in twelve hour periods. Differentiated subpopulations were depleted from the heterogeneous mixture with time and the undifferentiated hematopoietic stem cells increased in numbers.

The cells in the control groups in unit gravity and those in the bioreactors in conditions of microgravity were monitored under a number of parameters. Each were phenotyped as to cell surface antigens using a panel of monoclonal antibodies and flow cytometry. Other parameters compared included: pH, glucose uptake, oxygen consumption and carbon-dioxide production. Nuclear DNA was monitored by flow cytometry. Functional responses were studied by mitogenic stimulation by various lectins.

The importance of these findings should have relevance to the space program. Cells should behave predictably in zero gravity; specific populations can be eliminated from diverse populations and other populations isolated. The availability of stem cell populations will enhance both bone marrow and gene transplant programs. Stem cells will permit developmental biologists study the paths of hematopoiesis.

INTRODUCTION

This research is a study of murine bone marrow cells cultured in simulated microgravity. It has been shown by several investigators that gravity is an environmental factor which affects growth and functionality of cells. (Lorenzi, G., et al., 1986). Human lymphocytes exposed in microgravity to the mitogen Concanavalin A showed less than 3% of the activation of similar cells at unit gravity. (Cogoli, et al., 1984). The same investigators found that microgravity depresses and hypergravity enhances cell proliferation rates. These effects were particularly strong in cells undergoing differentiation. The cellular proliferation rates of several different cell types were increased by 30% in hypergravity while the consumption per cell of glucose was lower than at unit gravity. (Tschopp, 1983).

We have undertaken this research encouraged by the work of these investigators and others who have demonstrated that a microgravity environment alters cellular behavior. We have observed murine thymus cells and hybridomas under microgravity and we report this separately. Of particular interest to us is bone marrow for reasons of the benefits that can immediately be realized by discriminate elimination of subpopulations and by the isolation of precursor cells. We estimated that bone marrow cells contain a population of cells that are essentially fetal tissue and these should proliferate indefinitely while the differentiated cells that were produced from these would have finite division numbers. Furthermore, we anticipated that the minimization of cell-cell contact and the in vitro control of many stimulating factors would increase the accomplishment of the goals.

The availability of the bioreactors developed at the Johnson Space Center gave the initial impetus to this project. We were presented with the possibility of introducing the conditions of microgravity into the study.

The study of bone marrow under conditions that could alter subtype proliferation rates and functionality has particular relevance in a number of obvious areas. Developmental biologists may be able to elucidate pathways of hematolymphoid and myeloid differentiation. The removal of cancer cells from bone marrow for autologous transplants against neoplasia or removal of T cells for allografts in severe immunodeficiencies and in prevention of graft vs host effects might be accomplished more efficiently. If human pluripotent hematopoietic stem cells could be unambiguously identified and isolated, gene inactivation therapy might be advanced. (Weissman, et al., 1988).

If it is found that with time specific subpopulations are favored while others are not, we would attempt to sort and study these specific subsets within the bone marrow using the Fluorescence Activated Cell Sorter (FACS).

Since significant variance in the parameters studied in simulated hypogravity are evident from this research, we will propose that these in vitro investigations be repeated at zero gravity on a future space mission. We would ask that the study be undertaken not only with mice cells but with human cells.

The quest for the isolated clone of human hematopoietic stem cells has not been fruitful up to the present. We would like to accomplish this goal as part of a future space mission project.

MATERIALS AND METHODS

Mice. BALB/CANN HSD 4-5 week old, female, were used. These were purchased from Trudeau Institute, Saranac Lake, New York.

Cells. Bone marrow cells were obtained from the mice by flushing the tibiae and femora of ten mice with Hanks Balanced Salt Solution (without phenol red) supplemented with 2% Fetal Calf Serum. The cells were cultured at two million cells/ml Complete Medium: RPMI-1640, 1 mM Glutamine, 100 U/ml penicillin, and 10% Fetal Calf Serum. The Fetal Calf Serum was heat inactivated at 56 deg C for 30 min. The cells were kept at 37 deg C in an atmosphere containing 5% carbon-dioxide, 95% humidity.

Monoclonal Antibodies. The panel of antibodies included the following from clones purchased from American Type Culture Collection, Rockville, MD: TIB-146, (Anti B Cell, antiB cell precursors with antigen B220), TIB-207 (anti-L3T4 expressed on T helper-inducer subsets), TIB-150 (Anti Lyt 2.2, expressed on T suppressor-cytotoxic cells); TIB-120 (Anti Ia-b,d,q haplotypes); TIB -128 (Anti-MAC-1, macrophages and granulocytes). The following were from Sigma Chemical Company, St. Louis, Mo.: FITC-anti Thy 1.2 (cell surface differentiation antigen), and the secondary antibodies FITC-anti Rat IgG and FITC Goat anti-mouse. Obtained from Becton-Dickinson Co., Mountain View, CA: Anti-mouse Lyt-1 and anti-mouse I-A. MoAb E13 161-7, (anti-stem cell antigen) was a gift of Dr. Shelly Heimfeld of Stanford University. MoAb 2.4G2, (anti mouse Fc-receptor) was a gift of Dr. Mary Ann Principato of the National Institutes of Health, Bethesda, Maryland.

Staining. The conjugated monoclonals were used in direct staining. The unconjugated antibodies were incubated with the bone marrow cells and followed by the fluoresceinated the secondary antibodies. In each procedure the cells were not fixed but were incubated with a solution of propidium iodide which enabled us to sort out dead cells for flow cytometry analysis.

Simulated Microgravity. The bioreactor vessel used in our research was designed by the Engineering Department of the NASA-Johnson Space Center. The bioreactors are kept in incubators at 37 deg C, 5% carbon-dioxide, humidified atmosphere.

Flow Cytometry. Experimental data from the control cell populations grown in static culture at unit gravity and from the cells grown in microgravity in the bioreactor were obtained using Coulter Electronics EPICS V Cell Sorter (Coulter Electronics, Hialeah, FL). 10,000 events were scored on each test. Dead cells and cell aggregated were gated from the cells under study. For DNA analysis one ml suspension of cells were fixed with 2 ml 70% ethanol for 30 minutes and then incubated with a solution of propidium iodide/RNase. Cell counts were either by hemacytometer or by Coulter Counter.

Proliferative assay. Mitrogen stimulation was determined with Concanavalin-A and Lipopolysaccharide. Cells were pulsed for 16 hours with 3-H thymidine. Incorporation of radioactivity was measured by liquid scintillation counting. Cells were harvested in a multisample harvester and the data displayed as mean counts per minute.

Oxygen consumption and carbon dioxide production were determined using a Corning 166, pH Blood Gas Analyzer. (Corning, Medfield, MA, 02052).

Glucose utilization was determined on a Beckman Glucose Analyzer 2, Becton-Dickinson (Fullerton, CA, 92634).

Immunological Reestablishment: Three sets of ten mice each were irradiated with 825 rads radiation at MD Anderson Hospital, Houston, TX. One set of the mice were kept as controls. A second set received fresh bone marrow injections. The test group were given 100 cells/mouse of the cells which had been circulating in the microgravity vessel for forty days. Records of animal viabilities were kept. When the animals were sacrificed the colonies from CFUs were counted.

RESULTS

Bone marrow cells in static culture in complete medium at 37 deg decreased in numbers until day five. The cells in the flask at that time were primarily adherent cells. By daily removal of adherent cells it was possible to continue the culture. We discarded the cells grown in static culture at this time since we found that we could not maintain the non-adherent population. (16-7)

Bone marrow cells in the bioreactors also decreased in numbers until approximately day twelve. At this time there was an explosive increase in cell numbers. The increased showed some fluctuation but this was correlated with oxygen or glucose deprivation or excess carbon-dioxide production. When the culture media was changed on alternate days there was a doubling of numbers. This growth rate has been observed with three experiments beyond day forty. (16-8)

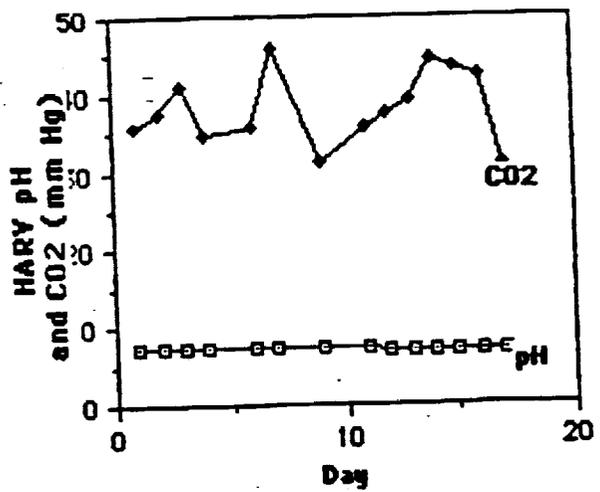
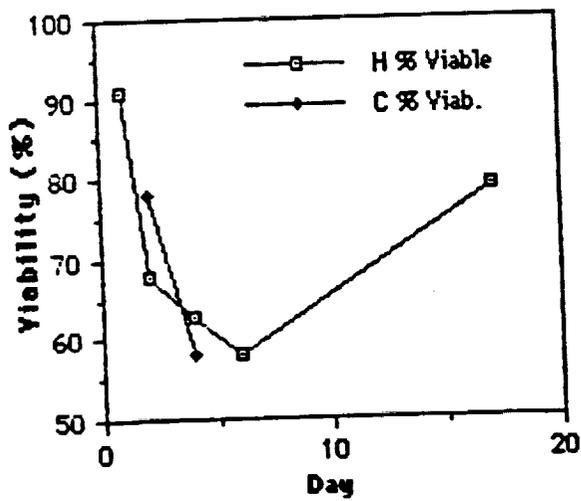
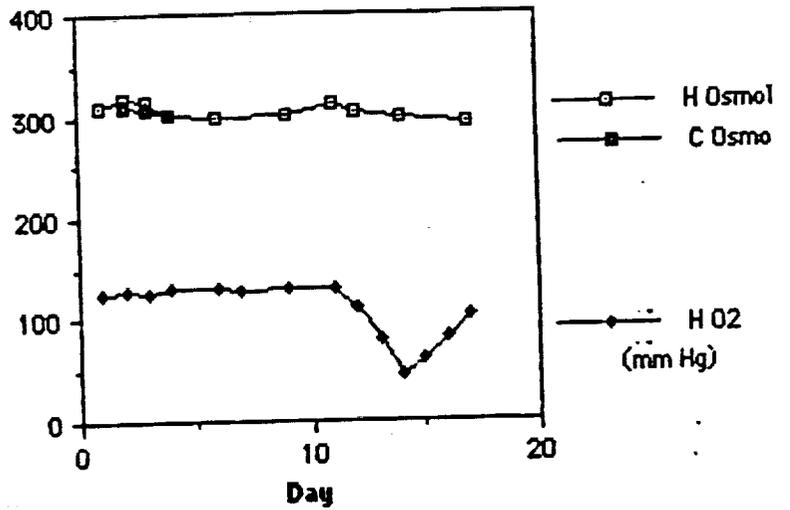
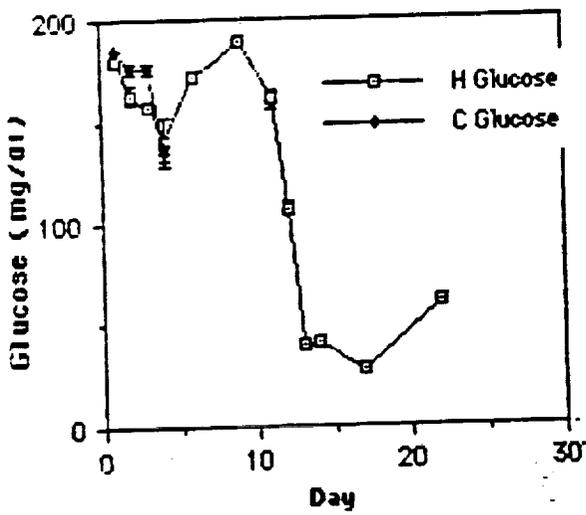
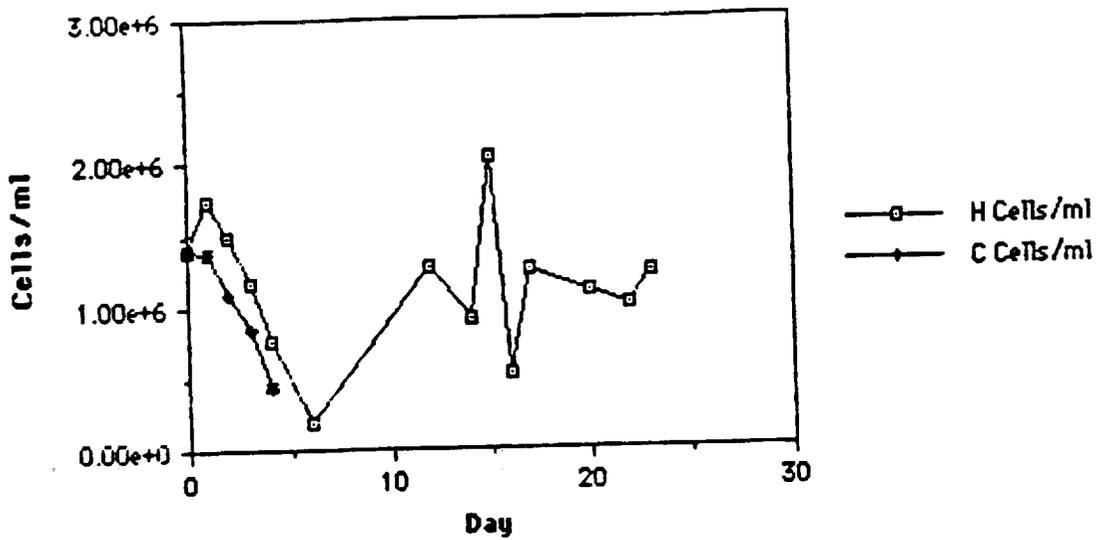
Condition of the cells was determined by monitoring glucose and oxygen use. Carbon-dioxide, pH and osmolarity also became useful parameters for estimating the condition of the cells. (16-9) Cell counts were determined by hemacytometer and viabilities were determined by trypan blue exclusion. (16-9)

Flow cytometric data was obtained only on live cells. Dead and damaged cells were gated by use of propidium iodide. (16-10). In the reprint listed, 69.43% of the cells (those in quadrant *4) were analyzed. DNA nuclear contents studied on lysed cells with propidium iodide was normal. It showed most cells in the 2N resting phase. (16-11).

Examples of the data obtained by flow cytometry is exemplified in 16-12, 13, 14). The cells in the bioreactors on day 26 were Thy-1 and SCA-1 positive and Fc-receptor negative.

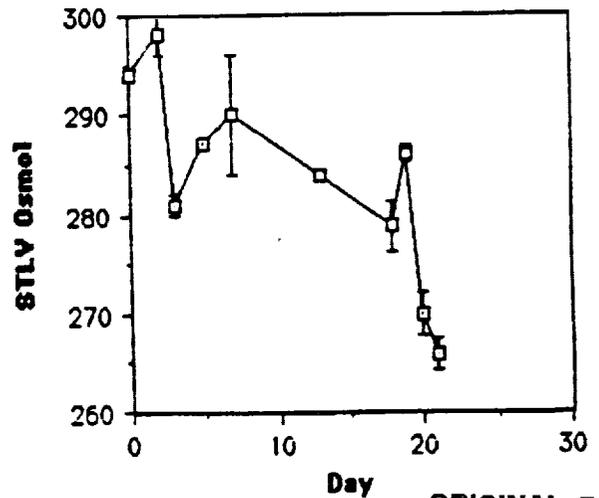
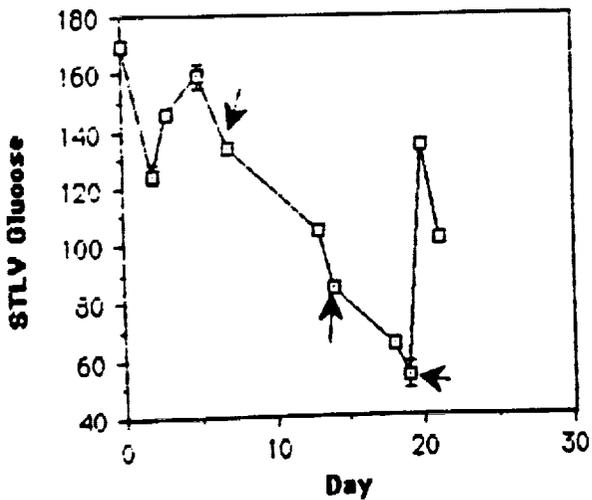
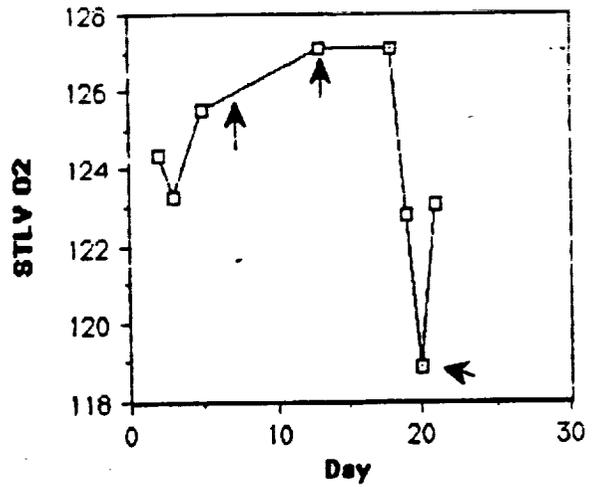
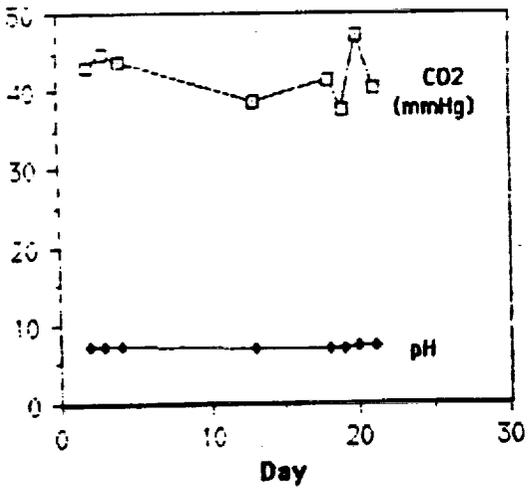
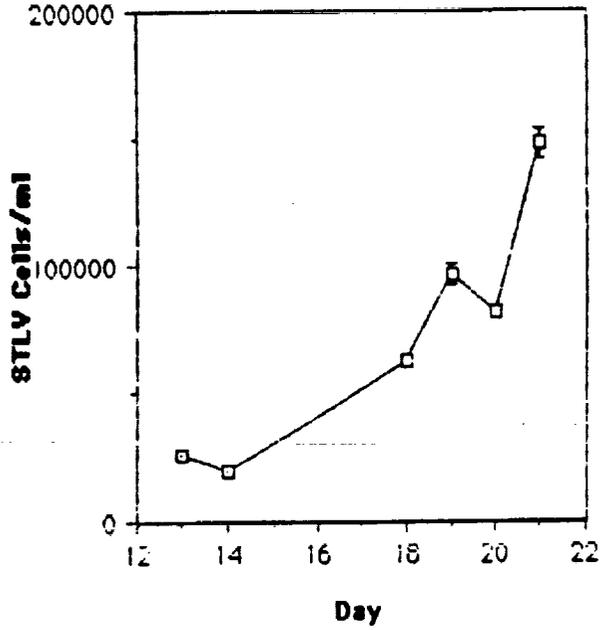
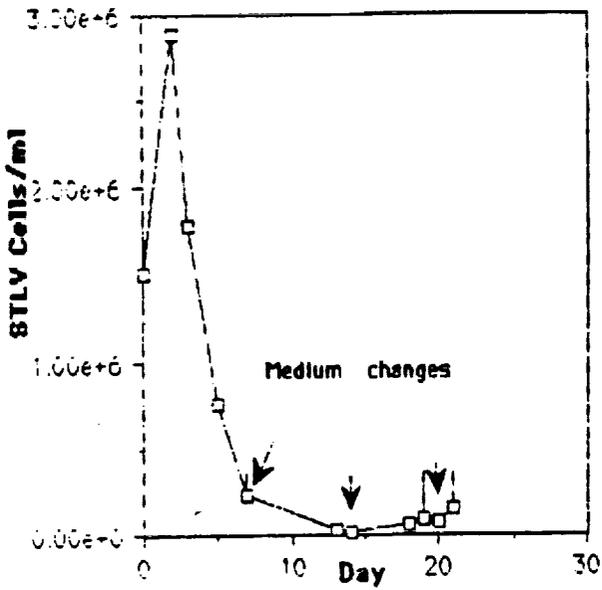
The ten mice which were given 900 rad lethal radiation and subsequent inoculation of bone marrow cells which had been growing in the microgravity environment of the bioreactor are all alive after six days. They will be continually monitored. A second group of four sets of lethally irradiated mice have been prepared. One set will receive an injection of normal bone marrow; a second will receive 100 bone marrow cells from the experimental vessels; a third set will receive amounts of cells ranging from one thousand to one million. The fourth set of mice will receive no cells.

6/19/89

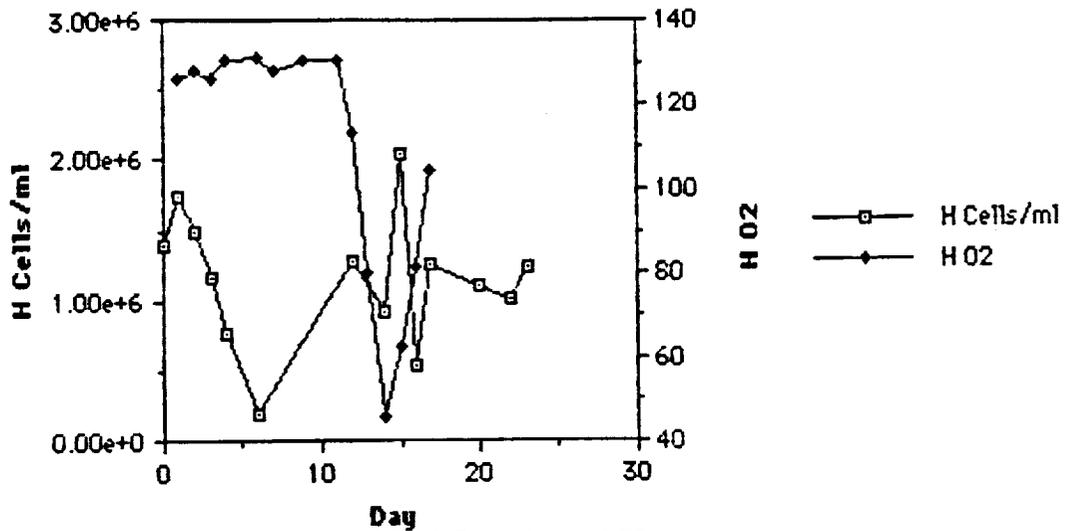


Test # 2 Bone Marrow 6/28/89 Delrin-end
STLV (Trudeau mice)

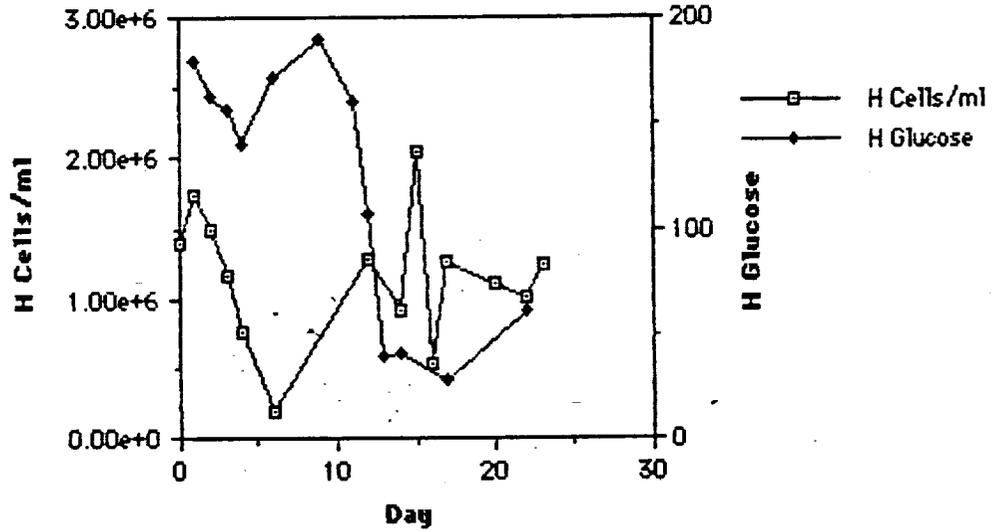
Selective Recovery of Cluster Type Cells



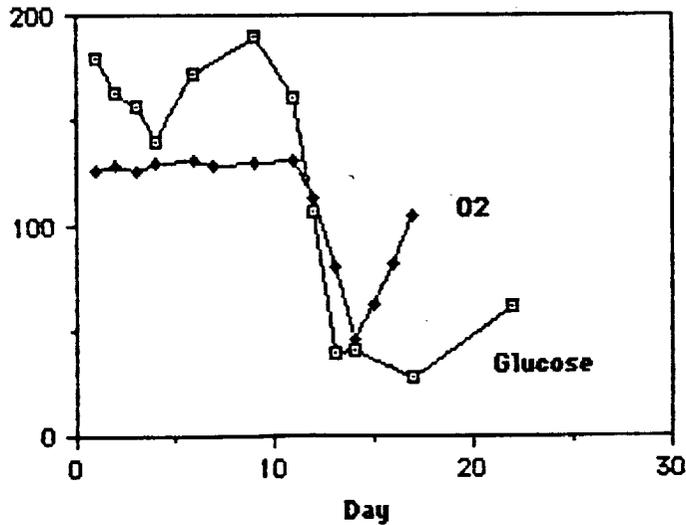
Relationship of Cell Growth and Oxygen Use



Relationship of Cell Growth and Glucose Utilization



Glucose and Oxygen Metabolism - HARY



ORIGINAL PAGE IS
OF POOR QUALITY

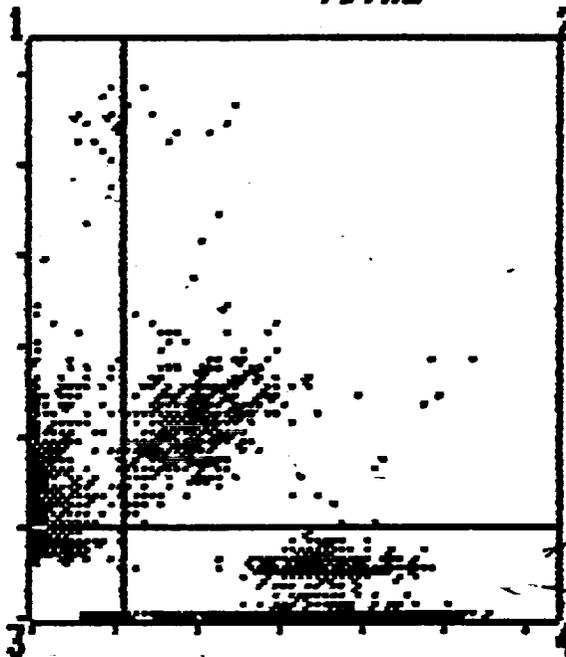
QUADRANT STATISTICS

LAN 14 0:50 20JUL89.00900E
 ← 831 GAM FITC
 FALS -LRFL

EXTENDED
 ANALYSIS

PRINT

TOTAL= 8544



FALS 11
 LRFL 10

LONEST LEVEL
 3

QUAD	PERCENT	PEAK POS	PEAK HT	AREA
1	9.89	0, 11	45	845
2	11.34	19, 21	13	969
3	9.34	0, 6	165	798
4	69.43	35, 0	383	5932

RETURN

READY.ADJUST CURSOR

31 days
DNA

ANALYZE
DATA

LAW 14
B31 PI DNA
IGFL /IGFL

3:29 28JUL89.06600C

CONT

SCALE 256

DISK
STORE

DISPLAY
BITMAP:
GATE

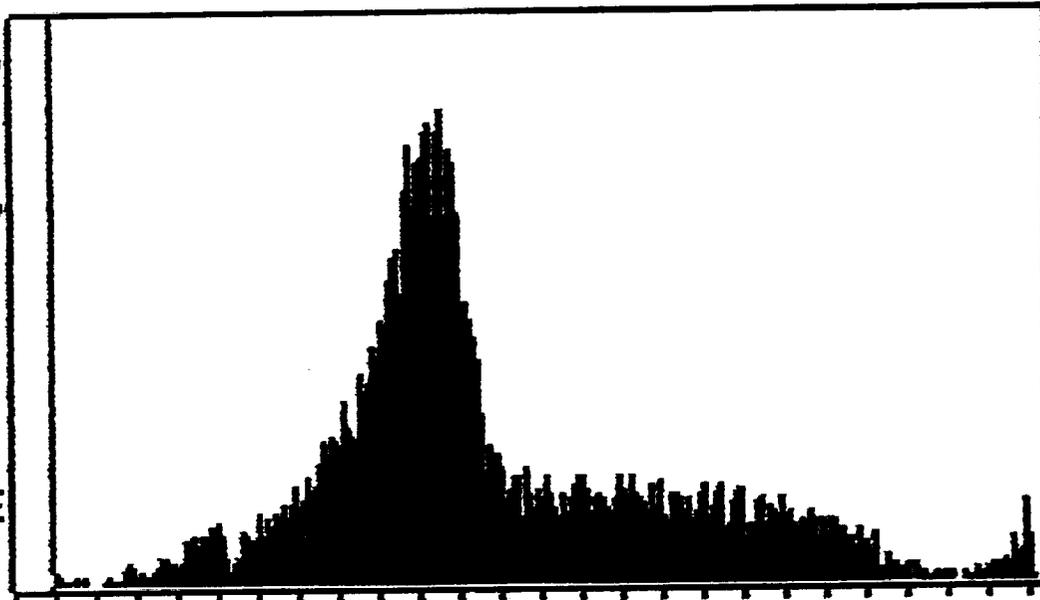
MEMORY
DELETE

DISPLAY
WINDOW:
GATE

RESTART

RESCALE

PLOT



IGFL GATE POSITION LOWER= 10 UPPER= 255

STATS

SEC

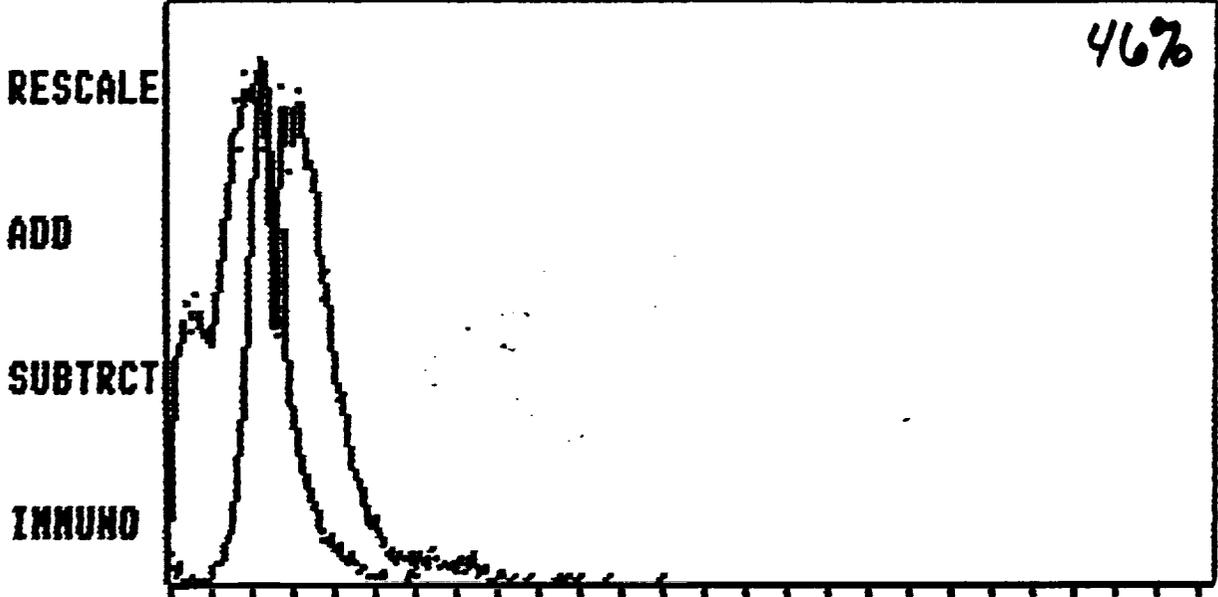
11
COUNT
10000

DONE

READY

TWO-HISTOGRAM COMPARISON

MERGE SCALE= 512 TOTAL= 10000
SCALE= 512 TOTAL= 10000



KSTEST LAW 16 8: 1 24JUL89.03400C
B26 THY1 N/AB
LGFL /LRFL ,FALS

LAW 16 8: 0 24JUL89.03300C
B26 GARFITC N/AB
LGFL /LRFL ,FALS

RETURN

READY

TWO-HISTOGRAM COMPARISON

MERGE

SCALE= 512
SCALE= 512

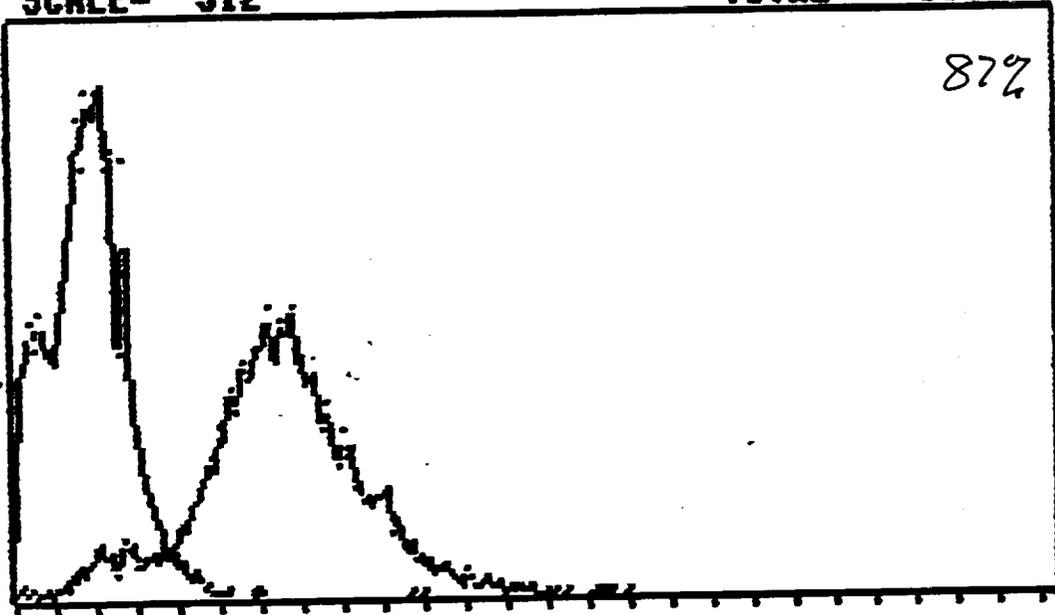
TOTAL= 10000
TOTAL= 10000

RESCALE

ADD

SUBTRACT

INNUHO



KSTEST

LAW 16 8: 5 24JUL89.03800C
B26 SCA W/AB
LGFL /LRFL ,FALS

LAW 16 8: 0 24JUL89.03300C
B26 GARFITC W/AB
LGFL /LRFL ,FALS

RETURN

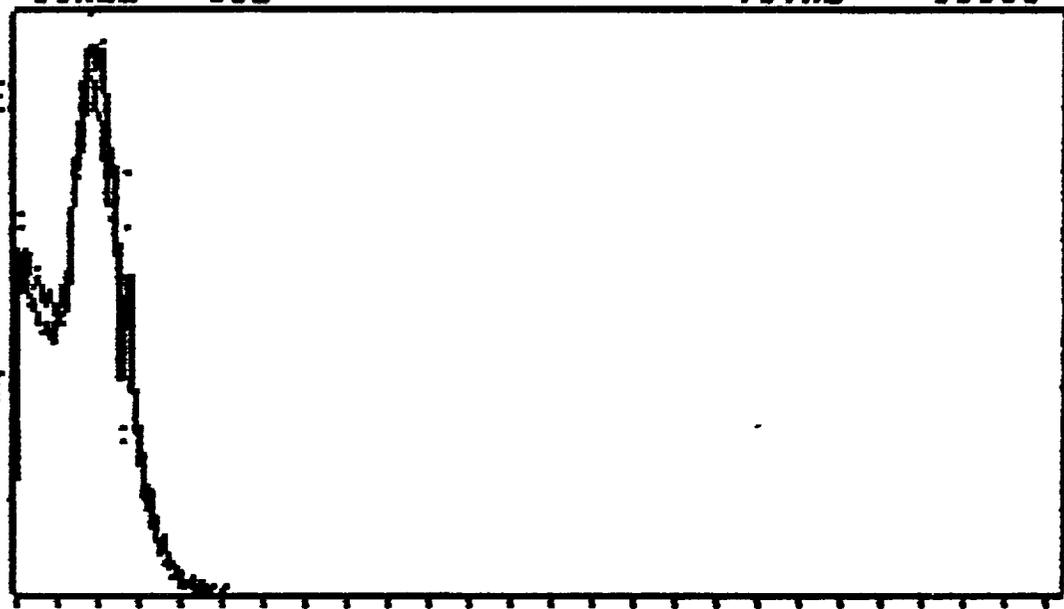
READY

FeR.

TWO-HISTOGRAM COMPARISON

MERGE SCALE= 512 TOTAL= 10000
SCALE= 512 TOTAL= 10000

RESCALE
ADD
SUBTRACT
INNUO



KSTEST LAW 17 5:52 25JUL89.03300C
B27 2462
LGFL /LRFL ,FALS

LAW 17 5:48 25JUL89.03000C
B27 GAR FITC
LGFL /LRFL ,FALS

RETURN

READY

DISCUSSION

Our objective in this summer research project was based on a number of premises. Cells of a fetal nature, e.g., bone marrow cells, would outlast differentiated cells if cell-cell contacts could be minimized. The in vitro separation would be discouraged because of the lack of cytokine secretion by cells other than the original population. The environments encountered in in vivo hematopoiesis would be missing. It was for these reasons that we turned to the microgravity environment made available by the bioreactor vessels designed at the Johnson Space Center.

The work has yielded many of the results that we had sought. It also left us with numerous questions that we wish to pursue. We must now look at human bone marrow, but first we must identify and isolate an antigenic marker for the human stem cell. We would then obtain a monoclonal to recognize this compound.

We are eager to continue observations which we have made this summer with the murine thymus and proceed to the human thymus. A third population of cells we would want to observe are fetal liver cells.

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